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# STUDIES ON THE HILL REACTION ACTIVITY OF SOLUBLE CHLOROPLAST EXTRACTS

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## Introduction

This study is concerned with the mechanisms and essential reactants of that part of the photosynthetic process in which oxygen is produced by photolysis of water. This is essentially the light absorbing reaction, which can be studied *in vitro* separately from the carbon dioxide fixing reactions. The photolysis of water and evolution of oxygen that occurs when chloroplasts or fragments of them are illuminated in the presence of a suitable electron acceptor is generally referred to as the Hill reaction. Attempts to reduce the Hill reaction system to a nonparticulate state have generally resulted in loss of activity. Progress toward this goal would be useful in defining the minimum components and conditions required for photolytic activity, as well as in determining the details of the mechanisms of energy transfer. Recent experiments along these lines in our laboratories led to establishment of this project to study chloroplast extracts.

## Experimental Plan

The long range plan of study includes analytical investigations to identify and characterize the components of the photoactive complex, and to determine the function of each; examination of the role of proteins, especially enzymes, in the complex; studies on methods of preparing extracts of chloroplasts, and the stability of the preparations; determination of the participation and role of various cofactors; and application of spectroscopic and other physical methods to elucidation of the characteristics of the system.

## Activity During the Period

The major areas of experimental investigation during this period included studies on methods of preparation and storage stability of chloroplasts and chloroplast fragments, use of digitonin in the preparation of active fragments and fractions, and attempts to improve Hill reaction activity of digitonin preparations by various procedures.

## Future Plan

Work during the next quarter will include studies on lyophilization as a means of preserving active preparations, other methods than digitonin digestion to fractionate chloroplasts, the role of plastiquinone and other cofactors, and the physical and chemical characteristics of the $S_3$ or similar fraction.

## Experimental

### A. Preparation and Storage Tests

Chloroplasts and fragments were prepared from fresh market spinach, typically as follows. The leaves were chilled, washed in distilled water, and deveined. They were then cut into small pieces and ground

with either a mortar and pestle or a Waring blendor, in a solution of 0.1M KCl, 0.5M sucrose, and 0.5M phosphate buffer of pH 6.6. The leaves were either illuminated or kept in the dark prior to grinding. The sucrose-phosphate buffer was used at the rate of 1 ml/g of leaf tissue. The leaves were ground only enough to liquify the preparation, then strained through four layers of cheesecloth containing one layer of glass wool.

The filtered suspension was centrifuged for 5 min at 2,000 rpm (480 g) to remove larger cell debris, then for 15 min at 3,000 rpm (1085 g) to collect whole chloroplasts, and finally for 15 min at 15,000 rpm (27,000 g) to collect chloroplast fragments. The pellets of chloroplasts or fragments were resuspended in sucrose-phosphate buffer, using a glass homogenizer. The sample preparation was all done under green light. Samples not used immediately for Hill reaction assays were frozen in 3-ml aliquots and stored in the dark in the freezer.

Hill reaction activity was determined by measuring oxygen evolution in the Warburg apparatus. In each test the Warburg flask contained: 0.2 ml 10% KOH in the center well; 0.8 ml pH 6.6 phosphate buffer, 1.0 ml 0.01M $K_3Fe(CN)_6$, 1.0 ml test sample in the side arm. Chlorophyll determinations were made on each sample by dissolving 0.5 ml in 25 ml of 80% acetone and measuring optical density in the spectrophotometer at 652 mμ: mg chlorophyll/ml = $\frac{OD_{652} \times 1000 \times 25}{34.5 \times 1000 \times 0.5}$. The oxygen evolution rate was calculated as: $Q_{O_2}^{Chl} = \frac{\mu l\ O_2/min \times 60}{mg\ chlorophyll/ml}$.

The effects of methods of preparation and storage periods tested so far are shown in Table I. Illumination and alternative methods of grinding appear to have relatively little effect on Hill reaction activity. The chloroplasts seem to keep a little better than the fragments during storage in the frozen state. Washing with large volumes of buffer did not seem to affect activity appreciably. In general, the preservation of activity in stored frozen samples was quite good. Studies on lyophilized samples are under way.

B. Effect of Digestion with Digitonin

Preparations were made as described above, through the first centrifugation at 2,000 rpm. The supernatant was centrifuged at 15,000 rpm, and the pellet was resuspended in 30 ml of sucrose-phosphate buffer with various amounts of digitonin added. The homogenized mixture was allowed to stand in the cold for 15 min, and was then centrifuged at 3,000 rpm for 10 min. The supernatant ($S_1$) was separated from the pellet ($P_1$), and a portion was centrifuged at 15,000 rpm for 15 min. The $S_2$ supernatant was separated from the $P_2$ pellet, and a portion was again centrifuged at 15,000 rpm for 60 min to prepare pellet $P_3$ and supernatant $S_3$. The pellets were resuspended in sucrose-phosphate buffer for Hill reaction measurement. Results are compared in Table II.

Table I

ACTIVITY OF CHLOROPLASTS AND CHLOROPLAST FRAGMENTS

| Preparation | Method of Grinding | Illumination | | $Q_{O_2}^{Chl}$ Days in Storage | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 0 | ½ | 1 | 4 | 7 | 13 | 24 | 29 | 49 |
| A | Mortar | Yes | Chloroplasts | 936 | | | | | | | | |
| | | | Fragments | 1361 | | | | | | | | |
| B | Blendor | Yes | Chloroplasts | 880 | | | | | | | | |
| | | | Fragments | 818 | | | | | | | | |
| C | Mortar[1] | No | Chloroplasts | 1064 | | 829[2] | | | | | | |
| | | | Fragments | 820 | | 469[2] | | | | | | |
| D | Mortar[1] | No | Chloroplasts | 661 | | 700 | | 600 | | | | |
| | | | Fragments | 591 | | 620 | | 500 | | | | |
| E | Mortar | No | Chloroplasts | | 1410 | | | | | | 1300 | 833 |
| F | Mortar | No | Chloroplasts | 900 | | | | | | | | |
| | | | Fragments | 600 | | | | | | | | |
| G | Blendor | No | Chloroplasts | 863 | | | 666 | | 783 | | | |
| | | | Fragments | 1091 | | | | | 545 | 600 | | |

[1] Washed with large volume of buffer after grinding

[2] Stored in cold, not frozen

Table II

ACTIVITY OF FRACTIONS OBTAINED WITH DIGITONIN TREATMENT

| Digitonin Concentration (%) | $Q_{O_2}^{Chl}$ | | | | | |
|---|---|---|---|---|---|---|
| | $P_1$ | $P_2$ | $P_3$ | $S_1$ | $S_2$ | $S_3$ |
| 2 | 395 | 882 | --- | 240 | --- | --- |
| 1 | 375 | 472 | 369 | 300 | 343 | --- |
| 1 | 960 | 555 | 274 | 106 | 143 | 125 |
| 1 | 291 | 350 | 392 | 292 | 111 | --- |
| 0.5 | 643 | 466 | 263 | 167 | 200 | 136 |
| 0.5 | 831 | 1181 | --- | 260 | 171 | --- |
| 1 | 584 | 1083 | 440 | 142 | 133 | --- |

C. Treatment of Digitonin Preparations

The experiments summarized in Table II suggested that the presence of digitonin might be reducing the Hill reaction activity, especially in the later supernatant fractions. Attempts were made to improve the activity by Sephadex chromatography and dialysis. The Sephadex columns were prepared with different grades suspended in 0.1M KCl. The sample was added to the top of the column and the column was drained until the sample was adsorbed. The column was then eluted with 0.5M phosphate buffer, pH 6.6.

On Sephadex 25, $P_3S_3$ formed two bands, one of which was eluted. $P_1P_3S_3$ together formed three bands, the first two of which were eluted. On Sephadex 50, $P_1P_3S_3$ together formed four bands, of which the first two were easily eluted, the other two not at all. On Sephadex 100, flow rates were very fast, and no separations were achieved. All of the eluted fractions contained digitonin, so the method was abandoned.

Two chloroplast preparations made as described above with 1% digitonin were used to investigate the effect of dialysis. In each case, after digestion with digitonin half of the sample was dialyzed in the cold against 3 liters of 0.5M phosphate buffer, pH 6.6, which was stirred mechanically during the dialysis. The other half of the sample was kept as a control at the same temperature. Both dialyzed and control samples were fractionated as described above. The results of Hill reaction measurements are shown in Table III. Dialysis for 4 hours did not effectively remove enough of the digitonin, while dialysis for 19 hours

Table III

EFFECT OF DIALYSIS ON ACTIVITY OF DIGITONIN EXTRACTS

| | $Q_{O_2}^{Chl}$ | | | | | | |
|---|---|---|---|---|---|---|---|
| | Whole Extract | $P_1$ | $P_2$ | $P_3$ | $S_1$ | $S_2$ | $S_3$ |
| Preparation #1 | | | | | | | |
| Before dialysis | 180 | | | | | | |
| 19-hour dialysis | ---* | --- | 165 | 270 | --- | 390 | --- |
| 19-hour control | 90 | --- | 180 | --- | 150 | 109 | --- |
| Preparation #2 | | | | | | | |
| Before dialysis | 231 | | $P_2$ —— $P_3$ | | | | |
| 4-hour dialysis | 240 | --- | — 250 — | | --- | | --- |
| 4-hour control | 90 | --- | 156 | | 150 | | --- |

* --- Indicates negligible activity

removed most but not all. Dialysis seemed to have a beneficial effect in some cases, though not consistently. The loss of activity in controls during storage at 4° C is discouraging toward the use of extended dialysis time.

Financial Status

During the second quarter, approximately 21 percent of allocated funds were spent. Work will continue at a slightly accelerated rate during the third quarter.

Respectfully submitted,

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